

Review Article

Regulation of EPCs: The Gateway to Blood Vessel Formation

Kate A. Parham,^{1,2} Stuart M. Pitson,^{1,3} and Claudine S. Bonder^{1,3,4}

¹ Centre for Cancer Biology, SA Pathology and University of South Australia, Frome Road, Adelaide, SA 5000, Australia

² School of Pharmacy and Medical Sciences, Division of Health Sciences, University of South Australia, Adelaide, SA 5000, Australia

³ School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, SA 5000, Australia

⁴ Centre for Stem Cell Research, Robinson Institute, School of Medicine, University of Adelaide, Adelaide, SA 5000, Australia

Correspondence should be addressed to Claudine S. Bonder; claudine.bonder@health.sa.gov.au

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Endothelial progenitor cells (EPCs) are primitive endothelial precursors which are known to functionally contribute to the pathogenesis of disease. To date a number of distinct subtypes of these cells have been described, with differing maturation status, cellular phenotype, and function. Although there is much debate on which subtype constitutes the true EPC population, all subtypes have endothelial characteristics and contribute to neovascularisation. Vasculogenesis, the process by which EPCs contribute to blood vessel formation, can be dysregulated in disease with overabundant vasculogenesis in the context of solid tumours, leading to tumour growth and metastasis, and conversely insufficient vasculogenesis can be present in an ischemic environment. Importantly, it is widely known that transcription factors tightly regulate cellular phenotype and function by controlling the expression of particular target genes and in turn regulating specific signalling pathways. This suggests that transcriptional regulators may be potential therapeutic targets to control EPC function. Herein, we discuss the observed EPC subtypes described in the literature and review recent studies describing the role of a number of transcriptional families in the regulation of EPC phenotype and function in normal and pathological conditions.

1. Introduction

The endothelial cell (EC) lined blood vasculature plays a vital role in maintaining vascular integrity, mediating pro- and anticoagulation and controlling immune cell trafficking (reviewed in [1, 2]). Precursors of ECs, namely, endothelial progenitor cells (EPCs), are integral contributors to vascular repair and neovascularisation [3], maintaining vascular homeostasis. Vasculogenesis is the process by which EPCs contribute to *de novo* blood vessel formation and is originally thought to only occur prenatally. However, in 1997, Asahara and colleagues identified EPCs in the adult, thereby revealing a role of these cells in postnatal vasculogenesis [4, 5]. Briefly, during vasculogenesis, bone marrow (BM) resident EPCs are mobilised into the circulation [5, 6] in response to EPC-activation factors which are upregulated in the circulating blood in response to hypoxia and vessel damage [7, 8]. Once in the circulation, EPCs traffic to sites in need of blood vessel formation and/or repair where they contribute to vasculogenesis (reviewed in [2]) by (1) an autocrine

process of differentiating into a mature EC and incorporating into the vasculature [9, 10] or (2) a paracrine process by secreting proangiogenic factors [11, 12]. Although EPCs are important mediators of physiological vasculogenesis, their dysregulation has been observed in pathological conditions. Reduced numbers and function of EPCs have been detected in patients with type-2 diabetes mellitus (T2DM, reviewed in [13, 14]) and cardiovascular disease (CVD, [15] and reviewed in [2]). Of note, these patients experience ischemic events and have an impaired EPC response, culminating in impaired vasculogenesis and amelioration of ischemia [15–20]. Conversely, high numbers of EPCs have been detected in cancer patients [21], where they contribute to solid tumour vascularisation and growth [22, 23].

Abnormal EPC function has been attributed to the dysfunction of gene regulatory factors that are important in controlling cell homeostasis. These regulators include gene transcription factors to specifically define cell types and cell states [24]. Here, we review the current literature on the subtypes of EPCs and investigate the signature of

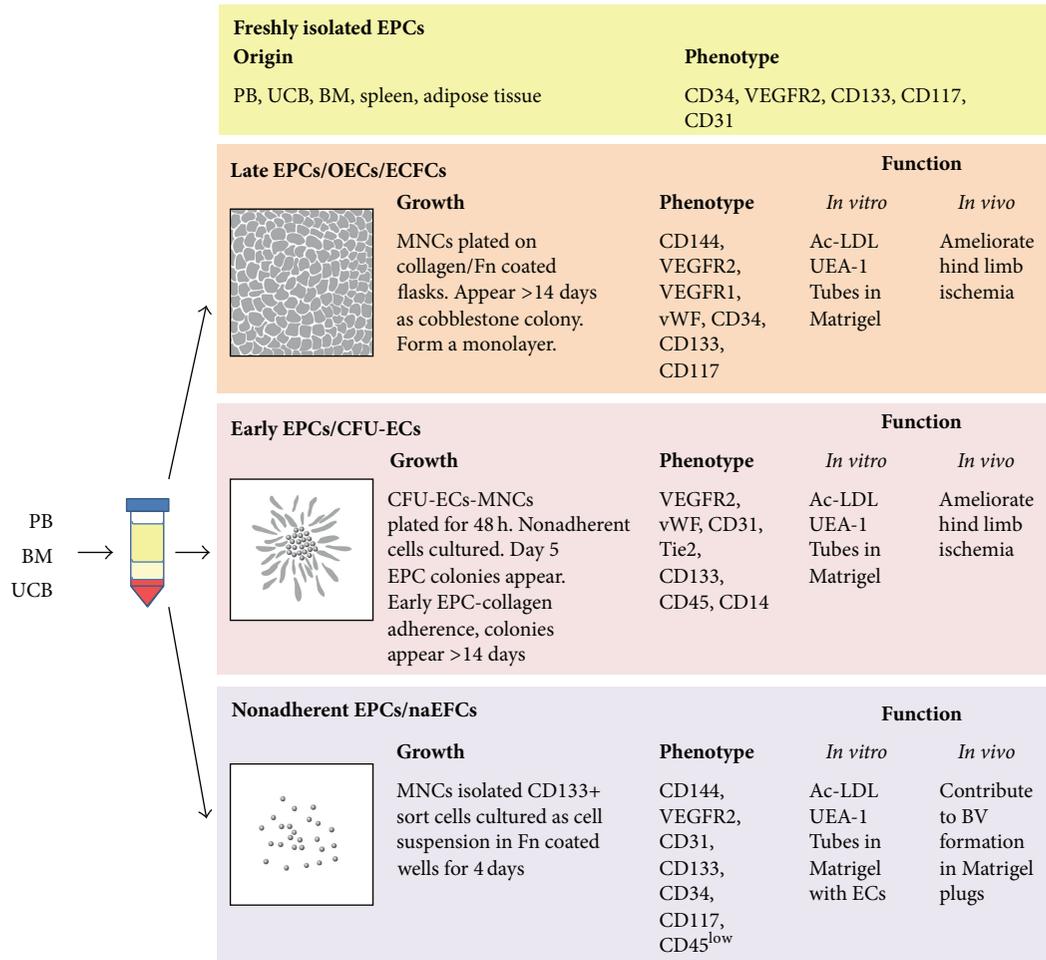


FIGURE 1: An overview of EPC subtypes. Comparison of freshly isolated EPCs with *in vitro* cultured EPC subtypes.

EPC transcriptional regulators and their roles in regulating phenotype and function of these cells in normal and disease states.

2. Endothelial Progenitor Cells

2.1. Human EPCs. EPCs were first identified in 1997 by Asahara and colleagues where they revealed a role of putative ECs in “therapeutic angiogenesis” [5]. In the following decades multiple groups have worked to further isolate and identify circulating EPCs in the human. Human EPCs can be isolated from a number of sources, including peripheral blood (PB) [5], umbilical cord blood (UCB) [25], BM [6], the spleen [26], and adipose tissue [27]. Following isolation, mononuclear cells (MNC) are separated by surface expressed proteins and enriched using various culture methods. As summarised in Figure 1, multiple cultured EPC subtypes have been identified with the most widely studied being an adherent population derived following 3-4-week cell culture. These endothelial-like cells are termed “late EPCs” [15, 28], “outgrowth ECs” (OECs) [29–31]), and more recently “endothelial colony-forming cells” (ECFCs) [32, 33]. Other adherent EPC populations are the short-term cultured “early EPCs” [15, 28] and “adherent colony forming unit-ECs” (CFU-ECs) [34].

Finally, more primitive nonadherent EPC populations have also been described [12, 35]. A detailed description of these cells is provided below. Notably, whether EPCs isolated from different tissues and organs can be further stratified into EPC subpopulations is yet to be fully elucidated and beyond the scope of this review.

2.2. Adherent

2.2.1. Late EPCs, Outgrowth ECs, and Endothelial Colony-Forming Cells. Late EPCs, OECs, and ECFCs are the most widely described EPC phenotype and will be referred to from here on as ECFCs (reviewed in [29, 36–39]). ECFCs can be derived from PB, UCB, and possibly also BM [29, 36, 37, 40–42]. The ability to isolate ECFCs from patients varies significantly with these cells phenotypically altered and reduced in number with age, presence of CVD, diabetes, and smoking [39, 43]. As summarised in Figure 1, ECFC populations arise after 2–4 weeks in culture and are characterised by a cobblestone colony which, over time, forms a monolayer [40]. ECFCs have a high proliferative potential compared to ECs [36, 39, 40] and as expected, ECFCs isolated from UCB generate more colonies and exhibit an increased proliferative rate compared to those isolated from adult PB [36]. Phenotypic

analysis shows ECFCs have strong expression of a number of endothelial markers including vascular endothelial-cadherin (VE-cadherin, CD144), vascular endothelial growth factor-(VEGF-) receptor 1 (Flt-1), VEGF-receptor 2 (VEGFR2, KDR, Flkl), endothelial nitric oxide synthase (eNOS), and von Willebrand factor (vWF) [36, 40]. A small percentage of these cells also express the progenitor markers CD34, CD133, and c-kit (CD117) [36]. Like ECs, ECFCs have also been shown to upregulate VCAM-1 expression in response to TNF α stimulation [36, 44]. Functional characterisation of these cells reveals that while they contribute to the amelioration of hind limb ischemia, like mature ECs, they are able to take up acetylated-low density lipoprotein (Ac-LDL), bind *Ulex europaeus* (UEA-1) lectin, and form capillary structures *in vitro* on their own [40]. Thus, ECFCs are largely indistinguishable morphologically, phenotypically, and functionally from mature ECs, and debate has arisen as to whether they are *bona fide* “progenitor” cells.

The origin of ECFCs is still unknown, with speculation as to whether they arise from circulating ECs, BM-derived cells, or a thus far unknown precursor [29, 37, 39, 41]. Initial studies by Lin and colleagues sought to establish what cell type was responsible for endothelial outgrowth from blood, utilising sex-mismatched BM transplantation [29]. This investigation revealed that the ECFCs arose from circulating angioblasts arising from the BM [29]. BM resident multipotent adult progenitor cells (MAPCs; CD45⁻/CD133⁻/CD34⁺), characterised in 2002 by Reyes et al., are suggested to be the progenitor of angioblasts. Upon treatment with VEGF these MAPCs differentiate the endothelial lineage and could be the precursor for ECFCs [45]. Interestingly, Timmermans et al. could not generate BM- or CB-derived ECFCs from CD133⁺ or CD45⁺ hematopoietic precursors [31]. Contrary to the evidence for the BM-derivation of ECFCs [29, 31, 45], a recent study suggests that these cells are readily isolated from UCB and PB but not BM [41]. Using surface antigen enrichment and depletion, the authors revealed ECFCs were confined to a CD34⁺/CD133⁻/CD146⁺ cell population. Of note, this population did not have hematopoietic potential in a colony forming assay and could not be detected in BM-MNCs [41]. One reason for the varied observations of ECFC origin may be due to the ambiguity of the ECFC phenotype. Regardless of their origin, once ECFCs appear in culture they have already acquired a phenotype directly comparable to mature ECs, lacking progenitor status and plasticity. Another contentious issue is that they arise over weeks of culture and are thereby likely to be somewhat altered from their native state.

2.2.2. Early EPCs and CFU-ECs. Early EPCs [15, 28, 40] or CFU-ECs [34, 46, 47] are isolated from total MNCs cultured on fibronectin in endothelial culture media. As depicted in Figure 1, these EPC subsets arise 7–14 days after isolation as colonies which are comprised of an apical cluster of rounded cells surrounded by spindle-shaped cells radiating from the centre [34]. Phenotypically early EPCs and CFU-ECs express the endothelial markers, CD31, VEGFR2, vWF, and Tie2; the progenitor markers, CD133 and CD45; and the monocyte markers, CD14 and CD11b [28, 34, 46, 48, 49]. Functional analysis of these colonies reveals a capacity to bind UEA-1

lectin and to take up Ac-LDL [15, 28, 40, 47]. The function of early EPCs and CFU-ECs *in vivo* has been investigated by a number of groups. Studies have shown that therapeutic administration of early EPCs improved blood flow and capillary density in a model of hind limb ischemia [28, 40] and confirmed incorporation of EPCs into the new vasculature [40]. The proangiogenic ability of CFU-ECs was elucidated in a solid tumour model using the lung cancer cell line A594 alone or with cotransplantation of CFU-ECs [46]. Tumours derived from A594 cells alone were shown to be half the size of those formed with cotransplantation of CFU-ECs. Notably, A594 tumours had a distinct necrotic centre and reduced vascularisation compared to A594 + CFU-EC tumours, suggesting that CFU-ECs contribute to neovascularisation *in vivo* [46]. Indeed, patients with cardiovascular risk [34] or coronary artery disease (CAD) [15] have reduced numbers and functional impairment of their CFU-EC population which may contribute to impaired vasculogenesis in these patients. Interestingly, the identity of early EPCs and CFU-ECs has been a point of contention, with a number of groups proposing they are monocytic [48, 49]. A recent study has supported this, suggesting that CFU-ECs are misidentified and are in fact predominantly CD14⁺ macrophages which have some endothelial characteristics [50]. The ambiguity of EPC identity has been discussed extensively [37, 39] and has been raised again more recently by Padfield and colleagues [50]. Importantly, CFU-ECs have confirmed proangiogenic function and therefore elucidating factors which regulate these actions remain of interest.

2.3. Nonadherent EPCs. Nonadherent EPCs have been described in a number of studies. We and others have isolated a nonadherent EPC population from the CD133⁺ MNC population of UCB and PB [12, 35]. We have described a population of nonadherent EPCs termed nonadherent endothelial forming cells (naEFCs [12]). naEFCs are cultured for 4 days on fibronectin in endothelial growth media complete with bullet kit and supplemented with 10% serum and additional growth factors (Figure 1) [12]. Janic et al. expanded CD133⁺ MNCs in serum-free defined nonadherent culture conditions and then transferred the progenitors into endothelial culture conditions [35, 51]. These naEFCs and CD133⁺ EPCs express CD31 and VEGFR2 in addition to being able to take up Ac-LDL and bind UEA-1 lectin and contribute to *in vitro* tube formation with ECs [12, 35]. The naEFCs were also shown to express the progenitor markers CD133, CD34, and CD117 in addition to being positive for the endothelial marker CD144 [12]. In addition, naEFCs express low levels of CD45 [12]. Importantly, naEFCs are CD11b and CD14 negative, excluding myeloid lineage and were not positive for the microparticle marker CD41a confirming the absence of microparticle contamination [12, 52]. Functionally, naEFCs were also shown to contribute to *in vivo* neovascularisation in a Matrigel plug assay [12]. Ahrens and colleagues isolated CD34⁺ progenitors from UCB which once transferred from expansion culture to endothelial conditions expressed CD144 and VEGFR2 in addition to the integrins β_2 (CD18) and β_3 (CD61) [51]. The cells were also shown to contribute to tube formation when cocultured with ECs in Matrigel and

bind UEA-1 lectin and take up Ac-LDL [51]. These expansion and differentiation studies may mimic what is seen *in vivo* whereby EPCs are recruited and come into contact with an extracellular milieu which dictates their differentiation and incorporation at sites of vascular repair or formation. Together, these studies have revealed the presence of non-adherent progenitors with distinct endothelial potential and as such may represent a *bona fide* EPC population in the circulation.

2.4. Rodent EPCs. There have also been extensive studies using EPCs isolated from rats and mice to investigate EPC biology. The primary source of EPCs from rodents has been from whole or sorted BM and to a lesser extent PB and spleen MNCs [20, 44, 53–60]. Similar to the human system, a unique identification signature for murine EPCs still remains elusive, with reported phenotypic overlap with macrophages and dendritic cells combined with inconsistent EPC phenotypes in different rodent strains [20]. One proven method to isolate murine EPCs from whole BM is through culture in endothelial specific media (EGM-2) on fibronectin for 7–10 days [20, 44, 56]. We have shown that EPCs adhere within 24 hours; and over 7–10 days these cells form colonies consisting of a mass of rounded cells surrounded by elongated cells radiating out from the centre which with extended culture form an EC monolayer [44]. Upon therapeutic injection of these EPCs into a mouse kidney ischemia reperfusion injury (IRI) model, they differentiate into mature ECs contributing to revascularisation [44]. Furthermore, we have isolated rat EPCs using a similar protocol where after 48 hours of culture the nonadherent cell fraction gave rise to EPC colonies which expressed CD34, Flk1 (VEGFR2), and CD31 [57]. Loomans and colleagues compared murine BM- and spleen-derived EPCs and revealed that spleen-derived EPCs showed lower proliferative potential than BM-derived EPCs [20]. As a whole, BM contains a heterogeneous population of cells and groups have isolated specific subsets of cells using flow cytometry or magnetic sorting [53–55, 58]. For example, a lineage negative MNC population is derived via removal of cells expressing the markers, CD5, CD45R, CD11b, Gr-1, and Ter119 [44, 53, 55]. These cells are then further enriched for progenitor cell markers, CD117 and Sca-1 [44, 53, 55]. Tsukada and colleagues compared murine EPCs isolated from PB-MNCs, BM-MNCs, and BM-CD117⁺/Sca-1⁺/Lin⁻ cells and observed that all three different starting cell populations gave rise to two types of EPC colonies (small and large) following culture in methylcellulose. Small and large colonies from all sources expressed the endothelial markers eNOS, Flk1, and CD144 [53]. Interestingly, the small EPC colonies contained a greater proportion of CD117⁺/Sca-1⁺/Lin⁻ cells and had greater proliferative potential but did not contribute to neovascularisation following hind limb ischemia, while the larger colonies exhibited improved tube forming capabilities *in vitro* and enhanced neovascularisation *in vivo* [53]. Furthermore, a study comparing CD34⁺, CD117⁺/Sca-1⁺/Lin⁻, CD117⁺/Lin⁻, and Sca-1⁺/Lin⁻ cells revealed that CD34⁺ cells had the lowest EPC colony forming potential in methylcellulose-containing medium but did express the highest levels of endothelial markers, vWF, CD144, and Flk1, and the homing molecules

integrin β_2 and CXCR4 [59]. Interestingly, *in vivo* CD34⁺ BM-MNCs exhibited enhanced recruitment, retention, and incorporation into the vasculature of ischemic myocardium when compared to CD117⁺/Sca-1⁺/Lin⁻, CD117⁺/Lin⁻, and Sca-1⁺/Lin⁻ cells [59]. A study by Sharpe III et al. has shown that culture expanded EPCs can also be isolated from a CD31⁺ myeloid cell population [58]. Together these studies support the notion that EPCs are present within a heterogeneous cell population of the BM and that even with enrichment via selection of CD117⁺/Sca-1⁺/Lin⁻ cells heterogeneity remains.

With this information in hand we will now focus on the transcriptional regulators and associated proteins of the various described EPC populations present within humans and rodents.

3. Transcription Factor Regulation of EPCs

3.1. Forkhead Transcription Factors. There are three members of the forkhead transcription factors (FOXO), FOXO1, FOXO3a, and FOXO4, with FOXO4 the most abundant [61, 62]. Under homeostatic conditions Akt actively retains FOXO transcription factors in a phosphorylated state which precludes them from translocating to the nucleus and results in their degradation [63]. By contrast, in their active form FOXOs are dephosphorylated and translocate into the nucleus for activation of FOXO target genes, such as the proapoptotic proteins, Bim, p27^{Kip1}, and Puma [64, 65]. With FOXO isoforms detected within human EPCs, these studies suggest that a balance between active and inactive FOXO transcription factors is vital for EPC survival [63]. To this end, a role of FOXO transcription factors in regulating EPC apoptosis has been identified and targeted in a number of pathologies. For example, the low circulating EPC numbers in CAD patients have been ameliorated with HMG-CoA reductase inhibitors (statins) which activate Akt causing FOXO4 phosphorylation and reducing EPC apoptosis (Figures 2(a) and 3(a)) [15, 62, 66, 67]. Conversely, scleroderma patients have significantly lower circulating CD133⁺ EPCs, due to enhanced apoptosis, correlating with reduced levels of active Akt and FOXO3a and resultant higher levels of the proapoptotic protein Bim (Figures 2(b) and 3(b)) [61]. Further support comes from a study by Alvarez et al. in which FOXO3a was identified as the contributing isoform for EPC survival in scleroderma patients [68]. Similarly, hyperglycemia impairs Akt activation driving FOXO1-mediated transcription of proapoptotic genes, Bim, FasL, p21, and p27 (Figures 2(b) and 3(b)) [18, 64, 65, 69]. Together, these data suggest FOXO transcription factors are integral in the regulation of EPC apoptosis and, subsequently, numbers in circulation. In addition to its effect on EPC survival, there is evidence to suggest that active FOXO3a also mediates early EPC differentiation, occurring in a time and phosphorylation dependent manner (Figure 4) [70]. Attenuation of Akt activity resulted in enhanced FOXO3a dephosphorylation in the early stages of EPC differentiation. This resulted in an increase in the number of EPC colonies, coupled with a reduction in Ac-LDL uptake and spindle-shaped cells, suggesting a role of dephosphorylated FOXO3a in maintaining an immature EPC phenotype [70]. During the final stages

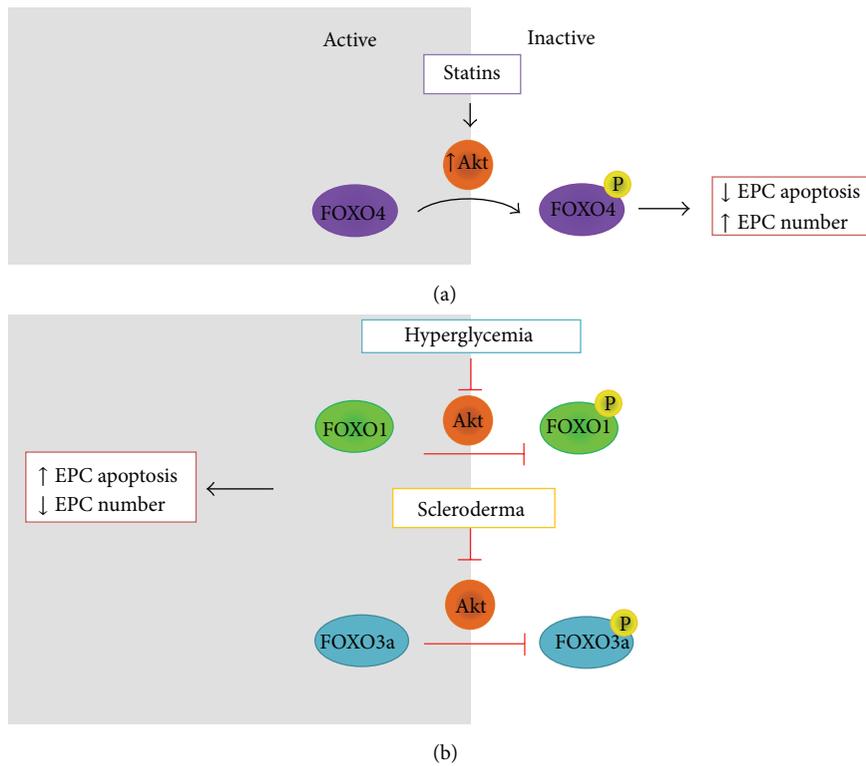


FIGURE 2: The FOXO family and EPC apoptosis. The balance between active and inactive FOXO dictates apoptosis in EPCs. Akt activity is central to this regulation with abundance of the phosphorylated inactive form of FOXOs resulting in reduced cellular apoptosis. Certain drugs and blood sugar levels can tip the balance of FOXOs in EPCs. (a) Statin treatment of patients with CAD stimulates Akt activity and in turn FOXO4 phosphorylation, culminating in reduced EPC apoptosis and amelioration of EPC number in circulation. (b) Moreover, hyperglycemia and scleroderma impair Akt activation and subsequent phosphorylation of FOXO1 and FOXO3a, respectively. This results in an abundance of active FOXO1 and FOXO3a leading to elevated EPC apoptosis and reduction in EPC number.

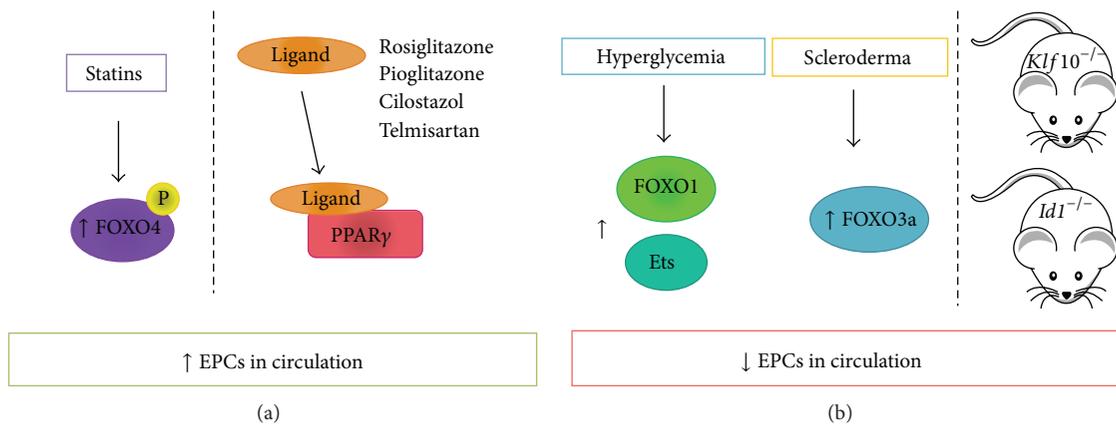


FIGURE 3: Transcriptional regulation of circulating EPC number. EPC numbers in the circulation are tightly regulated. (a) The reduced numbers of EPCs present in patients with CVD and T2DM can be ameliorated by current treatments. Statin treatment in CAD patients leads to reduced apoptosis via accumulation of phosphorylated FOXO4 leading to enhanced EPC number (Figure 2(a)). Agonists targeting PPAR_γ are used to treat T2DM (rosiglitazone and pioglitazone) and CVD (cilostazol and telmisartan). Systemic activation of PPAR_γ culminates in enhancement of EPCs in the circulation of patients. (b) The proapoptotic role of FOXO1 and FOXO3a (Figure 2(b)) contributes to reduced circulating EPCs in patients with hyperglycemia. Moreover, hyperglycemia correlates with increased activity in Ets resulting in reduced EPCs. In addition, mice deficient in *Klf10* and *Id1* have a documented reduction in EPCs in the periphery.

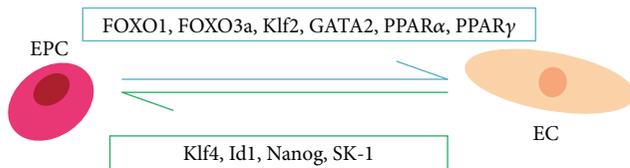


FIGURE 4: Regulation of EPC/EC state. The regulation of EPC/EC state is important as it can regulate cellular phenotype and function. There are a number of transcription factors which when active maintain EPCs in a progenitor state. They include Klf4, Id1, Nanog and SK-1 and when these are down regulated EPC phenotype and function can be altered resulting in EC differentiation. In addition, FOXO1, FOXO3a, Klf2, GATA2, PPAR α and PPAR γ have been shown to mediate the differentiation of EPCs to acquire characteristics of mature ECs.

of EPC differentiation and maturation into ECs, Akt activity gradually increased, allowing it to phosphorylate FOXO3a for its degradation [70]. In addition, hyperglycemic inhibition of EPC-mediated tube formation is reversed with benfotiamine treatment through restoration of FOXO1 activity and, like FOXO3a, EPC differentiation (Figure 4) [18, 70].

3.2. Kruppel-Like Factors. Kruppel-like factors (Klfs) are zinc finger, DNA-binding transcription factors. There are 16 family members (Klf1-16) which play diverse roles during cellular differentiation and development (reviewed in [71–73]). A role of Klfs in the vasculature has been revealed with EPCs documented to express Klf2, Klf4, and Klf10 [71]. Evaluation of ECFC transcriptional response to laminar flow revealed induction of Klf2 expression similar to that seen with mature ECs [74]. VEGF has also been shown to induce Klf2 expression, along with ECFC differentiation (Figure 4) [75]. Furthermore, implantation of murine BM-EPCs overexpressing Klf2 into mice in a Matrigel plug assay promoted vessel formation [75]. Interestingly, Klf4 appears to have an opposing but complementary role to Klf2 in the regulation of EPC differentiation. Reduced differentiation potential of ECFCs was evident following costimulation with VEGF and leukemia inhibitory factor (LIF), a member of the IL-6 family, due to Akt activation and a subsequent increase in Klf4 expression (Figure 4) [76].

Klf10 has been identified in more primitive proangiogenic cells (PACs) derived from murine common myeloid progenitors (CMPs) and granulocyte-macrophage progenitor cells (GMPs) [77]. Stimulation of CMPs and GMPs with TGF β enhanced Klf10 expression, thus promoting the generation of PACs through transcriptional upregulation of Flk1 expression [77]. Klf10 deficient mice (*Klf10*^{-/-}) exhibit a defect in the number of circulating PACs (Figure 3(b)) and reduced reendothelialisation which could be rescued by reconstitution of wild type BM [77, 78]. Importantly, the wild type BM transplant also increased the number of Lin⁻/CD34⁺/Flk1⁺ progenitor cells detected in circulation and at the site of revascularisation [78]. Of note, *Klf10*^{-/-} PACs exhibited reduced Flk1 and CXCR4 expression which may contribute to these effects as CXCR4 is critical in the mobilisation of EPCs from their BM niche and neutralisation of CXCR4 is known to reduce EPC mobilisation [77–79].

In addition, conditioned media from *Klf10*^{-/-} PACs do not support the growth of ECs while that from cultured wild type PACs do [78]. Taken together, these studies support a role of Klf10 in promoting proangiogenic cell differentiation, recruitment, and paracrine function which contribute to neovascularisation in response to ischemia.

3.3. GATA Transcription Factors. GATA-binding proteins (GATA) are a six-member zinc finger transcription factor family [80]. GATA-1, -2, and -3 are known to be expressed in the hematopoietic cell compartment, with GATA2 pivotal in the regulation of hematopoietic stem cells and progenitors [80]. Within hematopoietic cells GATA2 is a member of a stem cell leukemia (Scl) transcriptional complex which plays an essential role in hematopoietic development by enhancing CD117 expression (Table 1) [81]. Interestingly, together GATA2 and Scl have been shown to regulate ECFC phenotype, whereby stimulation of CD117 by its ligand, stem cell factor (SCF), increases ECFC neovascularisation [82]. CD117 is known to be upregulated in naEPCs compared to their EC counterparts [12, 82] and correlates with concordant increased expression of GATA2 and Scl in EPCs [82].

The dedifferentiation of mature ECs into a more immature state is another way by which EPCs can be identified, isolated, and manipulated [44, 83]. Coordinated gene expression of the nuclear transcription factors, GATA2 and c/EBP α , plays a role in endothelial function and cellular dedifferentiation of ECs. GATA2 regulates transcription of the endothelial marker CD31 [84], with CD31 downregulation strongly correlated with the loss of GATA2 and c/EBP α [85]. The silencer octamer-binding transcription factor- (Oct-) 1 has been shown to be a suppressor of the EC markers CD31 and vWF, through its action on c/EBP α and GATA2, culminating in EC dedifferentiation (Table 1 and Figure 4) [85]. These data suggest that, like FOXO1 and FOXO3a, within EPCs GATA2 plays an important role in regulating EPC differentiation and mature EC phenotype and may be manipulated to alter progenitor status [18, 70, 85].

3.4. Inhibitor of DNA-Binding. Inhibitor of DNA-binding (Id) proteins consists of four members (Id1-4) which belong to the basic helix-loop-helix transcription factors and lack DNA-binding to function as a negative regulator of gene transcription [86]. Id1 is a regulator of cellular proliferation and differentiation with its expression dysregulated in EPCs during disease [86]. For example, EPCs isolated from type-1 diabetic patients and patients with ovarian cancer have elevated expression of Id1 compared to healthy controls [87–89]. Moreover, in ovarian cancer this increase in Id1 has been linked with increased levels of integrin α 4 (Table 1) and PI3K/Akt-induced EPC mobilisation, recruitment, and survival, likely contributors to the progression of the cancer [88, 90]. Conversely, short hairpin RNA suppression of Id1 results in EPC mobilisation defects and severe reduction in tumour angiogenesis and growth [91]. This observation is supported by *Id1*^{-/-} mice as they are tumour resistant due to failure of BM-derived cells to mobilise from their BM niche (Figure 3(b)) [92]. In particular, Flk1⁺ endothelial precursors from *Id1*^{+/-}*Id3*^{-/-} mice have impaired response to VEGF

TABLE 1: Regulation of EPC phenotype.

	Endothelial markers	Adhesion molecules	Progenitor markers	References
Stimulation				
GATA2	↑CD31, vWF		↑CD117	[81, 85]
Id1	↓CD144, vWF		↓CD133, CD34	[96]
PPAR α	↑CD31, CD144	↑ICAM-1		[106]
PPAR γ	↑CD144, vWF	↓ICAM-1, VCAM-1		[116, 121]
Nanog	↓CD31, CD144, vWF			[134, 136]
Inhibition				
Id1		↑ Integrin α 4		[88]

vWF: von Willebrand factor; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1.

which has been shown to upregulate Id1 expression [93–95]. Furthermore, loss of Id1 in the BM results in elevation of the cyclin dependent kinase inhibitor, p21, resulting in cell cycle arrest and loss of the circulating murine EPC population (Lin⁻/CD117⁺/Flk1⁺), contributing to a reduction in tumour growth via reduced neovascularisation [92]. Interestingly, the observed reduction in circulating EPCs in *Id1*^{-/-} and *Id1*^{+/-}*Id3*^{-/-} mice is similar to that seen with *Klf10*^{-/-} mice [77, 78]. Id1 has also been shown to mediate the effects of secreted extracellular matrix component CCN1, present within the local vascular environment, which regulates murine EPC adhesion, proliferation, and *in vitro* capillary formation [96]. Following vascular injury Id1 expression is reduced in response to CCN1, stimulating differentiation of EPCs into ECs at the site of injury (Figure 4). Localised differentiation in response to Id1 downregulation was determined by an increase in EC markers (eNOS, vWF, and CD144) and a reduction in progenitor cell markers (CD133 and CD34), contributing to vascular regeneration at the site of vessel repair (Table 1) [96]. Moreover, these observations implicate Id1 in the maintenance of the EPC population. These studies suggest that Id1 functions differently depending on cell type and location.

With an emerging role of Id1 in regulating EPCs in both physiological and pathological processes, Id1 expression has been exploited as a method to track EPCs in BM, blood, and tissue stroma [91]. In a study by Mellick and colleagues, Id1 was used as a selective marker for EPCs directing the delivery of the suicide gene, herpes simplex virus-thymidine kinase, to abolish EPC mediated vasculogenesis and, in turn, tumour growth [91]. Other Id proteins have been detected in ECFCs; they include Id2 and Id3, with Id3 downregulation occurring in response to TGF β and contributing to ECFC transdifferentiation into smooth muscle-like cells [97]; further studies to support these findings are yet to be undertaken.

3.5. E26 Transformation Specific Sequence (Ets) Transcription Factors. There have been 30 Ets transcription factors identified in humans which are known to play essential roles throughout embryonic development and in adult life via the regulation of hemopoiesis and angiogenesis. Dysregulation of Ets has also been detected in cancer (reviewed in [98]). Within early EPCs levels of Ets1 and Ets2 are tightly regulated to control normal EPC numbers [99, 100]. T2DM, however, appears to result in dysregulated levels of Ets transcription

factors in early EPCs [99]. Indeed, hyperglycemia induces Ets activity in EPCs resulting in reduced EPC numbers, similar to FOXO1 [18], and impaired EPC function with an inability to commit to the endothelial lineage *in vitro* (Figure 3(b)) [99]. This may be mediated by p38 MAP kinase as this signalling molecule is located upstream of the Ets transcription factors and has been shown to play a role in reduction of EPC proliferation and differentiation in patients with T2DM [100]. Importantly, inhibition of Ets transcriptional activity rescued hyperglycemia-induced reduction in EPC number and these cells were observed to have restored endothelial lineage commitment; with normal EPC function restored these EPCs have the ability to contribute to the amelioration of cardiovascular defects, common in patients with T2DM [99].

3.6. Peroxisome Proliferator-Activated Receptors. Peroxisome proliferator-activated receptors (PPARs) are transcriptional members of the nuclear receptor superfamily. PPARs facilitate transcription as a complex, existing as heterodimers with retinoid-x-receptors [101, 102]. In response to ligand activation coactivator proteins are recruited, driving nuclear translocation and binding of PPAR response elements in the target gene promoter to mediate gene expression [103]. The PPAR family contains three members, α , β/δ , and γ , which are major players in adipogenesis and metabolism (reviewed in [103–105]). A role of PPAR α in regulation of EPC function has not been extensively investigated. One study has, however, shown that microparticle PPAR α mediated differentiation of murine BM-derived EPCs (Figure 4) [106]. Briefly, treatment of EPCs with microparticles containing PPAR α resulted in increased expression of proangiogenic and endothelial markers (CD31, CD144, and intercellular adhesion molecule-1 (ICAM-1), Table 1), in addition to enhancing capillary-like structure formation *in vitro*. Increased tubule formation was also detected *in vivo* in a Matrigel plug assay [106].

Activation of EPCs by the synthetic PPAR β/δ agonist, GW501516, has been used to identify a role of PPAR β/δ in EPC biology. Initial studies looking at early EPCs revealed that PPAR β/δ agonist treatment increased EPC viability, proliferation, and migration mediated by the PI3K/Akt pathway [107]. Furthermore, systemic activation of PPAR β/δ in mice led to increased EPC numbers in the periphery [107, 108]. In two animal models, human early EPCs treated with GW501516 accelerated limb reperfusion by increasing

capillary density and increased corneal neovascularisation [107]. These effects were shown to be mediated by the stimulation of matrix metalloproteinase-9 (MMP-9) secretion from EPCs leading to proteolysis of insulin-like growth factor (IGF) binding protein 3 [108]. This cleavage releases free IGF to activate IGF receptors promoting angiogenesis and was shown to enhance wound healing in a mouse skin punch wound model [108]. In ECFCs, activation of PPAR β/δ contributes to cell proliferation and VEGF-induced migration *in vitro* by stimulating the biosynthesis of tetrahydrobiopterin, an essential cofactor of nitric oxide synthase (NOS) [109]. Furthermore, in a model of carotid artery injury in nude mice, activation of PPAR β/δ enhanced the ability of ECFCs to repair the endothelium [109].

Of the three isoforms, PPAR γ has been the most extensively investigated in EPC biology, mainly utilising synthetic PPAR γ agonists such as thiazolidinediones (TZD) which include rosiglitazone, pioglitazone, and troglitazone [110]. As mentioned previously, patients with T2DM exhibit EPC dysfunction. Interestingly, similar to statin induced increase in phosphorylated FOXO4 [66, 67], treatment of patients with rosiglitazone induces a significant increase in the number and revascularisation capacity of early EPCs, independent of glycemic control (Figure 3(a)) [111, 112]. This phenomenon was also apparent with pioglitazone treatment of patients with CAD (Figure 3(a)) [113]. In response to rosiglitazone, increased numbers of CD144⁺/CD31⁺ cells are evident on the surface of injured vessels, while pioglitazone treatment increased the number and function of Scal⁺/Flk1⁺ EPCs in PB and BM [114, 115]. *In vitro* pioglitazone treatment led to enhanced early EPC and ECFC viability, enhanced *in vitro* tube formation and reduced expression of the inflammatory mediators TNF α , vascular cell adhesion molecule-1 (VCAM-1) and, in contrast to PPAR α , reduced ICAM-1 [106, 116]. Importantly, inhibition of PPAR γ reduced expression of these inflammatory mediators (Table 1) [116]. Supporting an anti-inflammatory role of PPAR γ , Verma and colleagues showed that C-reactive protein-dependent impairment of EPC number and function was ameliorated with rosiglitazone treatment [117]. Furthermore, H₂O₂- and TNF α -induced apoptosis of EPCs have been shown to be inhibited by pioglitazone and rosiglitazone treatment, respectively [113, 118, 119]. Consistent with these findings, activation of PPAR γ has been shown to enhance EPC numbers. In murine EPC models, PPAR γ activation has been shown to promote differentiation of angiogenic progenitor cells toward the endothelial lineage (Figure 4) [114, 115]. Telmisartan, a partial agonist of PPAR γ , has also been shown to increase nonadherent EPC number by increasing proliferation via stimulation of PI3K signalling and subsequent reduction in p21 activity (Figure 3(a)) [120]. Furthermore, early EPCs treated with telmisartan experience an increase in EPC number, proliferation, and migration toward VEGF, in addition to enhanced expression of CD144, vWF, and eNOS (Table 1) [121]. Interleukin-3 (IL-3) is another molecule that promotes EPC proliferation via PPAR γ . In a study by Dentelli and colleagues, IL-3 activated the transcription factor STAT5 in CFU-ECs promoting increased expression of PPAR γ and formation of a STAT5/PPAR γ

heterodimer, which regulated cell cycle progression by controlling cyclin D1 expression [122]. This phenomenon was not seen via activation of PPAR γ with TZDs or the PPAR γ agonist, palmitic acid, which instead led to cell cycle arrest due to a lack of cyclin D1 transcription [122, 123]. These data suggest that PPAR γ mediated proliferation can be modulated differently depending on the composition of ligand in the cellular environment. The vasodilating antiplatelet drug cilostazol has also been shown to activate PPAR γ and, like TZDs, increase circulating EPC number and enhance EPC function, including proliferation, adhesion, and differentiation (Figure 3(a)) [124–126]. In a rat model of balloon carotid artery denudation, rats given cilostazol for two weeks after surgery exhibited accelerated reendothelialisation with an increased number of BM-derived EPCs detected at the injured luminal surface and reduced neointima formation compared to control [124].

These studies confirm PPAR γ as a critical regulator in the maintenance of EPCs in both normal and disease states. In summary, not only does PPAR γ regulate EPC function (e.g., proliferation and apoptosis) but it also plays an integral role in regulating EPC numbers within the circulation and can be modulated to increase PB EPC number [111–113, 124].

3.7. Nanog. Nanog is a member of the homeobox family of DNA-binding transcription factors [127] and in a dose-dependent manner maintains pluripotency in ES cells derived from human, monkey, and mouse by regulating genes associated with self-renewal, such as SRY-related HMG-box (Sox) 2 and Oct4 [128–131]. Within the last ten years a role of Nanog in EPCs has also been revealed. High expression of Nanog in early EPCs isolated from PB (CD14⁺/CD34^{low}/VEGFR2⁺) [132] or UCB (CD45⁺/Lin⁻) [133] has been described, with the differentiation into ECs coinciding with downregulation of Nanog (Figure 4). Interestingly, the PB- and UCB-EPCs in these studies were multipotent, as they could also differentiate into osteoblasts and neural and muscle cells [132, 133]. Fibrin is a known inducer of Nanog expression leading to increased early EPC cell viability and a reduction in mature endothelial markers of CD144, CD31, and vWF (Table 1) [134]. Furthermore, ECFCs isolated from PB were analysed for the expression of self-renewal genes; Nanog was not detected which is not unexpected as these cells are largely indistinguishable from mature ECs further pointing to a role of Nanog in maintaining the primitive status of EPCs, similar to the roles of Klf4 and Id1 [76, 96, 135]. Notably, there is increasing evidence to support agonist-induced enhanced Nanog expression in mature ECs. First, we recently showed that overexpression of sphingosine kinase-1 (SK-1) in ECs dedifferentiated these cells towards an EPC phenotype and that this coincided with an induction of Nanog at the mRNA and nuclear protein level [83]; second, Kohler and colleagues showed that stimulation of ECs with Wnt3A resulted in increased transcription of Nanog and subsequent expression of Flk1, contributing to enhanced EC proliferation and angiogenesis [136]. Taken together these studies suggest that expression of Nanog in mature ECs leads to dedifferentiation and induction of stemness, implicating it as an important player in maintaining progenitor phenotype.

TABLE 2: EPC derived from cellular reprogramming.

	iPS cells	IVPCs	SK-1	Epigenetic modification
Starting cell type	Mouse/human embryonic fibroblasts, ECFCs	Rat ECs	HUVEC	Murine and human EPCs
Intervention	Retroviral transduction of Oct4, Sox2, Klf4, c-Myc	Lentiviral transduction of Oct4, Sox2, Klf4, c-Myc	Lentiviral transduction of SK-1	Inhibitors of DNA methyltransferases (5-azacytidine), histone deacetylases (valproic acid), G9a histone dimethyl-transferase (BIX-01294)
Dedifferentiation	Full	Partial	Partial	Partial
Phenotype	↑Alkaline Phosphatase, Sox2, Oct3/4, Nanog High E-cadherin	Low E-cadherin	↑CD34, CD133, CD117 ↑Nanog ↓CD144, CD31, vWF	Enhanced global transcription ↑Oct-4, Nanog, Sox2 ↑eNOS, CD144
<i>In vitro</i> function	↑Proliferation Form embryoid bodies Can differentiate into neural and cardiac cells	Form tubes <i>in vitro</i> Align to flow Differentiate into ECs in response to VEGF	↓Ac-LDL uptake ↓ <i>in vitro</i> tube formation	
<i>In vivo</i> function	Form teratomas	Improve coronary artery flow and cardiac function in a repeated MI model Do not form teratomas		Treated EPCs improved ejection fraction, left ventricular function; reduced infarct size, left ventricular fibrosis in a MI model Do not form teratomas
References	[137–139]	[141]	[44, 83]	[143]

iPS: induced pluripotent; iVPC: induced vascular progenitor cells; SK-1: sphingosine kinase-1; HUVEC: human umbilical vein endothelial cell; MI: myocardial infarction.

4. Cellular Reprogramming

4.1. Induced Pluripotent Stem Cells. Cells with EPC function can be derived from fully differentiated somatic cells utilising the technology which produces induced pluripotent stem (iPS) cells. First evidence of this process was in 2006 where mouse embryonic fibroblasts (MEFs) and human fibroblasts were reprogrammed via retroviral overexpression of factors known to have a role in maintaining pluripotency [137, 138]. Briefly, 24 genes and factors were screened for inducing stem cell colony formation, revealing a combination of four specific factors required to induce a pluripotent phenotype in the MEFs. As summarised in Table 2, the four factors included Oct4 and Sox2, both involved in self-renewal and maintaining pluripotency, in addition to Klf4 and c-Myc, an oncogene required for proliferation [137, 138]. Expression of these transcription factors in somatic cells generates cells which express endogenous levels of stem cell markers, have a similar growth curve and morphology to stem cells, and are able to differentiate into all three germ layers and as such are termed iPS cells [137, 138]. A recent study has differentiated murine iPS cells into Flk1⁺/CD144⁺ ECs which are able to contribute to *in vitro* and *in vivo* vessel formation in Matrigel to a greater extent than mature ECs [136]. ECFCs isolated from MNCs have been used as a starting population for iPS cell generation [139, 140]. In these studies, Oct4, Sox2, Klf4,

and c-Myc (OKSM) were retrovirally expressed in ECFCs for reprogramming to a pluripotent state [139]. These ECFC-derived iPS cells were identified to have a greater potential for differentiation and vascular regeneration; however, a potential for teratoma formation was observed [137, 138].

4.2. Induced Vascular Progenitor Cells. Yin and colleagues have looked to generate an induced vascular progenitor cell population. Rat ECs transduced with OKSM can be reprogrammed to a progenitor cell type that is committed to an endothelial specific lineage [141]. As described in Table 2, transduced ECs underwent the early stages of reprogramming; however, upon further analysis it appeared that these iPS cells expressed low E-cadherin, a protein essential at high levels for the formation of fully reprogrammed iPS cells [141, 142]. This suggests that the ECs were not completely reprogrammed and thus identified as induced vascular progenitor cells (iVPCs) [141]. The function of iVPCs was assessed and revealed an ability to form tube-like structures in Matrigel, aligned to shear flow, expression of the endothelial surface proteins CD31, CD144, and vWF, and importantly, the ability to differentiate into ECs in response to VEGF [141]. *In vivo*, iVPCs did not form teratomas and improved coronary collateral flow and cardiac function in a rat model of repeated myocardial ischemia [141]. Taken together, these studies reveal a role of partial reprogramming of vascular cells

in enhancing their vasculogenic capacity in the absence of some of the undesirable characteristics of iPS cells.

4.3. Sphingosine Kinase-1. In addition to pluripotency transcription factors, other factors have been shown to regulate progenitor status of vascular cells. Notably, SK-1, a signalling enzyme that generates the bioactive phospholipid, sphingosine 1-phosphate, is a regulator of EPC differentiation (Figure 4) [44, 83]. SK-1 activity was significantly elevated in murine BM-derived EPCs compared to their EC counterparts [44]. Comparing EPCs from wild type and SK-1 knockout mice we observed an increase in endothelial function (Ac-LDL uptake and tube formation in Matrigel), a reduction in the progenitor marker Sca-1, and an increase in CD144 expression when SK-1 was absent [44]. As depicted in Table 2, a subsequent study confirmed that overexpression of SK-1 in human ECs caused dedifferentiation toward a progenitor like phenotype with increased expression of the progenitor markers, CD34, CD133, and CD117, and modest downregulation of the endothelial markers, CD144, CD31, and vWF [83]. Importantly, SK-1 overexpression caused a reduction in endothelial function with reduced uptake of Ac-LDL, impaired tube formation in Matrigel, and elevated expression of the pluripotency transcription factor Nanog [83]. The induction of Nanog by SK-1 implies this lipid enzyme may maintain progenitor status and may contribute to regulating the roles of Klf4 and Id1 in this process. Together, these studies suggest that SK-1 is an important regulator of EPC differentiation and can be manipulated to alter progenitor status of vascular cells. Of note, manipulating SK-1 could not completely dedifferentiate ECs into EPCs, suggesting that a combination of factors, like that observed in iVPCs, may be required for complete reversion.

4.4. Epigenetics. Thal et al. have investigated whether manipulation of the epigenetic signature of mouse BM-derived EPCs and human CD34⁺ EPCs can enhance angiogenic potential [143]. As outlined in Table 2, treatment of Lin⁻/Sca⁺/CD31⁺ EPCs and human CD34⁺ EPCs with epigenetic modifying molecules, such as inhibitors of DNA methyltransferases, histone deacetylases, and G9a histone dimethyl transferases, removed epigenetic marks which restrict gene transcription, leading to an increase in expression of the pluripotency genes Oct4, Nanog, and Sox2 [143]. Interestingly, the expression of EC-specific genes was maintained in EPCs with epigenetic modification suggesting that these cells do not acquire pluripotency. Consistent with this, these cells did not form teratomas *in vivo* [143]. Furthermore, in an *in vivo* murine model of acute myocardial infarction, epigenetically reprogrammed human CD34⁺ cells exhibited enhanced therapeutic efficacy and paracrine activity [143].

5. Concluding Remarks

One difficulty in this field is the ambiguity in defining an EPC. To date there is no consensus on what constitutes an EPC by surface antigen expression alone. To date there are chiefly three distinct EPC subtypes in the literature: ECFCs, CFU-ECs or early EPCs, and naEFCs. These EPC

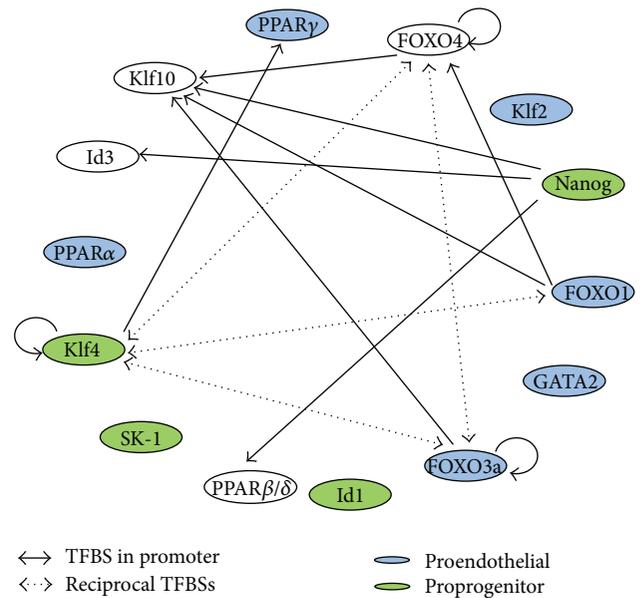


FIGURE 5: Predicted transcriptional regulatory network in EPCs. FANTOM4 freeware was used to generate a regulatory network of the EPC transcription factors discussed in this review by analysing transcription factor binding site (TFBS) predictions [144].

subtypes each have different progenitor characteristics, with naEFCs the most primitive and ECFCs similar to mature ECs; however, all have vascular characteristics including the ability to contribute to tube formation whether it is *in vitro* or *in vivo*, bind UEA-1 lectin, and take up Ac-LDL. Here we have shown that there are a number of different transcription factors which work together to dynamically regulate EPCs, for example, their abundance in circulation, apoptosis, phenotype, and EPC/EC differentiation. Furthermore, the cutting edge technology that gave rise to iPS cells is now emerging in the vascular biology field with the partial reprogramming of ECs to give rise to iVPCs, through gene overexpression or modulation of epigenetic markers. There is already evidence to suggest that targeting transcription factors, for example, PPAR γ , in EPCs enhances their numbers and function in circulation; however, one possible caveat is that transcription factors regulate a large number of target genes and therefore off target and adverse effects may occur and need to be closely evaluated. Moreover, as depicted in Figure 5, analysis of a predicted regulatory network of the transcription factors discussed in this review suggests that they may cooperatively regulate EPC phenotype and function. Therefore targeting one of these factors may in fact result in manipulation of a number of transcriptional pathways and warrants further in-depth analysis. EPCs play an important role in both physiological and pathological processes by contributing to vasculogenesis. The opportunity to modulate EPC function via targeting their transcriptional signature could lead to the amelioration of diseases, for example, by enhancing EPC angiogenic function in ischemic patients or the inhibition of EPC-mediated neovascularisation in those with malignancies, as depicted in Figure 6. Therefore further investigation

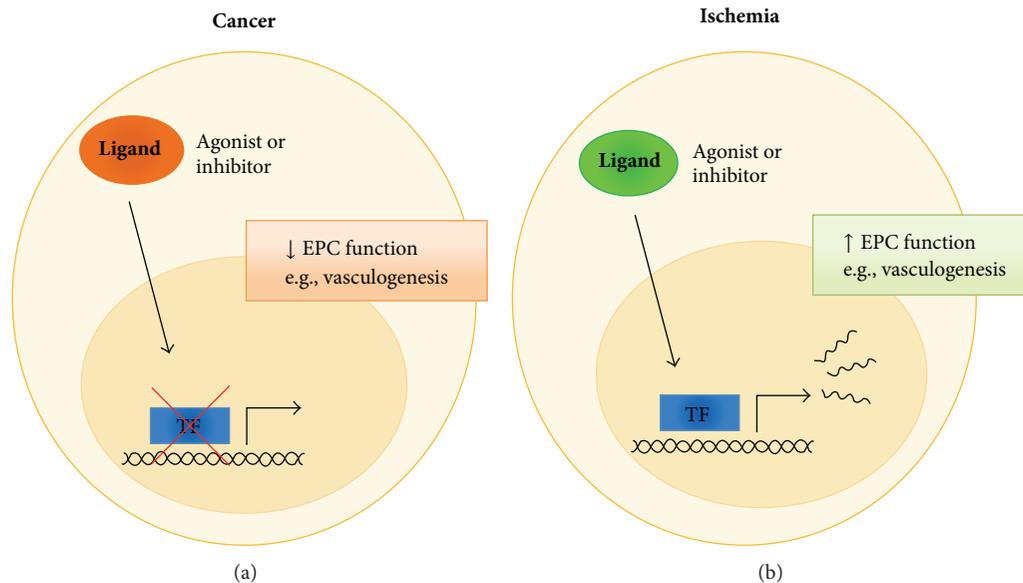


FIGURE 6: Schematic of potential therapeutic applications for targeting transcription factors in EPCs. EPCs are known to contribute to vasculogenesis in solid tumours as well as being dysfunctional in conditions which increase patients' susceptibility to ischemic events. (a) For example, in a tumour setting inhibiting transcription factors which are known to enhance EPC function and contribution to vasculogenesis may result in reduced tumour growth by limiting tumour vascularisation. (b) Conversely, in the context of ischemia, targeting transcription factors in EPCs to enhance their vasculogenic potential could contribute to the amelioration of ischemia.

into how EPCs are transcriptionally regulated may reveal pathways which can safely be targeted to alter EPC function in disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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